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Research Article

Can the Eurasian otter (*Lutra lutra*) be used as an effective sampler of fish diversity? Using molecular assessment of otter diet to survey fish communities

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Abstract

The Eurasian otter *Lutra lutra* is a generalist carnivore that is widely distributed in many aquatic ecosystems. Based on its inherent attributes of opportunistic foraging behaviour and broad dietary range, it is naturally considered a potential sampler of the diversity of aquatic vertebrates. To test the ability and efficiency of otters as a diversity sampler, we used DNA metabarcoding to investigate the composition in vertebrates of the diet of otters that inhabit a forest stream area in northeast China. Twenty vertebrate prey taxa were detected in 98 otter spraints. Otter diet mainly comprised aquatic fishes (59.4%) and amphibians (39.0%). We also used traditional approaches to investigate fish communities at 60 sampling sites in the same area to determine the relationship between fish population composition in the environment and otter diet. The comparison revealed that 28 species of fish were distributed in this area, of which five are simultaneously detected in otter spraints. This indicates that molecular analysis of the diet of otters is not an ideal approach for investigating fish diversity, at least when using the 12SV5 primer pair. Based on a review of the available molecular research on otter diet, we conclude that the low species resolution may be due to the presence of many closely-related prey species in native habitats and lack of suitable barcodes. Considering the remarkable power of diet metabarcoding analysis in capturing elusive and rare species, it represents an approach that can compensate for the defects associated with fishing methods and we suggest that it can be used as an auxiliary means of measuring traditional fish diversity.

Key Words

12S rRNA, diet, diversity sampler, DNA barcoding, species resolution, spraints

Introduction

Freshwater ecosystems represent one of the most diverse and dynamic ecosystems in the world (Vorosmarty et al. 2010; Thomsen et al. 2012). Due to a variety of anthropogenic factors (e.g. habitat degradation, contamination and pollution, overharvesting and the introduction of exotic species), freshwater species, particularly vertebrate species, have suffered the steepest decline, more so than marine or terrestrial species (Jelks et al. 2008; Reid et al. 2019). The rapid loss of biodiversity not only reduces the functions of ecosystems and the services they provide, but also affects society as a whole (Deiner et al. 2016). Accurate assessment of biodiversity and its changes is a critical step and an essential prerequisite for achieving reasonable and effective ecological protection and management (Pereira et al. 2013; Sato et al. 2017). However, traditional methods for investigating freshwater biodiversity, which depend mainly on capture-sampling and morphology-based taxonomic identification, are invasive, selective, time-consuming and labour-intensive (Kubecka et al. 2009; Zhang et al. 2020). Furthermore, these

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methods appear to be inefficient in capturing elusive, rare and highly mobile underwater animals (Jerde et al. 2011; Fujii et al. 2019). With advances in molecular biotechnology, detection techniques based on environmental DNA (eDNA) have broken through the limitations of traditional methods and provide an alternative, effective and cost-efficient tool for biomonitoring and biodiversity assessment (Thomsen et al. 2012; Bohmann et al. 2014; Hanfling et al. 2016).

Secretions and excretions produced by multifarious organisms are released into environmental media (water, soil and air), resulting in the presence of myriad types of eDNA in environmental samples. Appropriate deep sequencing of this eDNA can be used to identify constituent species and to rapidly assess ecosystem-level biodiversity (Taberlet et al. 2012; Rees et al. 2014). For example, to investigate fish diversity in freshwater ecosystems, eDNA is extracted from water samples; then, based on its composition and without prior isolation of the organisms, the species that produced it can be identified by the metabarcoding method using PCR primers that offer high species resolution with next-generation sequencing (NGS) (Miya et al. 2015; Valentini et al. 2016; Miya et al. 2020). This approach greatly improves the rate of detection of fish species and the efficiency of freshwater biodiversity evaluation and has led to considerable expansion of this field in recent years (Zhang et al. 2020; Consuegra et al. 2021). However, eDNA also has its own limitations that cannot be ignored, such as the significant spatial autocorrelation of water samples, especially in flowing water, resulting in difficulties in assessment of the geographical distribution of species (Eichmiller et al. 2014; Civade et al. 2016; Dickie et al. 2018). Besides, there is no direct connection between the reads of species determined by eDNA with the abundance or standing biomass of the respective individuals, which makes it difficult to accurately measure the species richness using eDNA (Bohmann et al. 2014; Zinger et al. 2019).

In this context, the use of faeces, another type of environmental sample, has attracted attention. Study of faeces has been widely applied in biological research fields, such as population genetics, studies of feeding habits, animal behaviour, determination of intestinal microorganisms and even pathogens (Klare et al. 2011; Deagle et al. 2013; Chakrabarti et al. 2016). Shao et al. (2021a) used faecal DNA metabarcoding analysis to investigate the feeding habits of two small carnivores, the red fox (*Vulpes vulpes*) and the leopard cat (*Prionailurus bengalensis*), in several mountainous areas in China and compared the results obtained in this way with the local species composition obtained by traditional survey methods. The two types of investigations revealed similar degrees of diversity. Shao et al. (2021a), therefore, proposed that generalist carnivores (generally small and medium-sized carnivores) that possess the inherent attributes of opportunistic foraging behaviour and broad diet range could be used as samplers for evaluating environmental biodiversity.

As a highly generalist predator, the Eurasian otter (*Lutra lutra*) is widely distributed in many aquatic ecosystems and occurs in relatively abundant populations worldwide (Kumari et al. 2019; Andersen et al. 2021); furthermore, its biological characteristics fully meet the diversity sampling criteria described above. In addition, Eurasian otters mainly feed on fishes and have high dietary plasticity that is related to resource availability in the habitat they occupy (Clavero et al. 2003; Remonti et al. 2008; Krawczyk et al. 2016). Therefore, they are potential samplers of fish diversity in aquatic ecosystems. More importantly, otters have fixed home areas (Hutchings and White 2000; Quaglietta et al. 2015; Quaglietta et al. 2019); this can effectively eliminate spatial autocorrelation problems in water eDNA analysis and has innate advantages for analysing species spatial distribution and structure analysis.

To determine whether the Eurasian otter can be used as a sampler for fish diversity surveys, the efficiency of molecular analysis of otter spraints for surveying environmental fish communities must be accurately evaluated; however, research in this field is still lacking. In addition, as the top predator in aquatic ecosystems, Eurasian otters play an important role in mediating the balance and stability of those ecosystems (Krawczyk et al. 2016; Zhang et al. 2023). The Eurasian otter is listed as a near-threatened species on the International Union for Conservation of Nature Red List (Roos et al. 2015) and it is also a class II endangered animal on the list of key protected wild animals in China (Zhang and Fan 2020). Knowledge of otter diet and whether otters consume potentially contaminated prey is critical in supporting efforts for their conservation.

In this work, we used a NGS-based DNA metabarcoding approach to investigate the diet and prey profiles of Eurasian otters inhabiting a forest stream area in northeast China. To validate the efficiency of otters as diversity samplers, we surveyed local fish communities with conventional methods. In addition, we reviewed existing studies on molecular dietary analysis of otters to explore the following questions: (1) Can the Eurasian otter be used as an effective fish diversity sampler? (2) What are the key factors that affect the efficiency of detecting individual species in otter diets? (3) What is the composition of otters' food and are there seasonal and regional differences? Additionally, we asked: (4) What are the prospects for molecular diet investigation?

Materials and methods

Study site

The study site is located within the Hunchun National Reserve (HNR) and surrounding areas (130°14'08"–131°14'44"E, 42°24'40"–43°28'00"N, Fig. 1) in north-eastern Jilin Province, China. The HNR, which has a total area of 1,078 km^2, has been under the jurisdiction of the Northeast Tiger and Leopard National Park since

2016. The geography of the HNR is mountainous, with high terrain in the north and low terrain in the south. The climate is a typical temperate continental monsoon climate with mean monthly temperatures ranging from -17.4 to 25.9 °C and a frost-free period of 120 ~ 126 days/year. Based on whether the ground was covered with snow, we divided the year into two seasons (snow-cover season, Nov-Apr and snow-free season, May-Oct). The mean annual rainfall in the study area ranges from 580–618 mm and most precipitation occurs in the summer from June to August. There are 52 large and small rivers in the HNR that together form a dense water network. The average river length per square kilometre is more than 4.3 kilometres, and the annual average run-off depth exceeds 400 mm. These conditions provide a suitable hydrological environment for Eurasian otters.

Faecal sample collection and DNA extraction

Faecal samples were collected weekly along the streams in the study area in January and May 2020 during the snow-cover season and the snow-free season, respectively (Fig. 1). To improve sampling efficiency and reduce the need for repeated sampling, we only collected fresh faeces surrounded by traces of otter activity at intervals of at least 200 m. The collected scats were clamped with disposable tweezers and placed in 50-ml tubes containing 95% ethanol. The samples were then transported to the laboratory and stored in a freezer at -20 °C until DNA extraction was performed.

To extract DNA from faecal samples, we used the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Inc, Hilden, Germany) according to the manufacturer's instructions with minor modifications as described by Shehzad et al. (2012). Approximately 250 mg of faeces from the external surface of each sample was used in DNA extraction for molecular species identification. The otter-positive faecal samples were manually homogenised using a disposable sterilisation scalpel and total DNA was extracted from each 250 mg of homogenised faeces and eluted in 100 µl of warmed elution buffer before being used in the molecular dieta'ry analysis. An extraction tube without sample was included in each batch of extractions to control for contamination during DNA extraction (i.e. extraction blanks).

Molecular identification of samples source

We used the primer pair Lutcyt-F/Lutcyt-R, which targets the partial mitochondrial cytochrome b gene (227 *bp*), to identify the spraint samples (Park et al. 2011). PCR was performed in a 15-µl volume containing 7.5 µl 1× Premix Taq (Takara, Inc, Dalian, China), 0.2 µM Lutcyt-F/R primer, 1 µg bovine serum albumin (BSA; TaKaRa, Inc, Dalian, China) and 20–40 ng DNA template. The PCR conditions were an initial denaturation step of 5 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 35 s at 55 °C, and 40 s at 72 °C and a final extension step at 72 °C for 10 min. Negative controls without faecal DNA were systematically performed to check for possible contamination. In addition, we also used DNA (0.08 ng/µl) extracted from the muscle tissue of a Eurasian otter as a positive control.

PCR products were Sanger sequenced on an ABI 3730XL automatic sequencer (PE Applied Biosystems, Inc, CA, USA) using the Big Dye Sequencing Kit. Sequences matching the sequenced fragments were retrieved using the NCBI BLAST programme. Samples that matched the Eurasian otter cytochrome b gene sequence (GenBank accession number KU953404.1) with ≥ 98% identity were identified and used in the subsequent dietary analysis.

Efficiency of molecular species analysis to assess otter diet

Restricted by the poor quality of faecal DNA, the barcodes used in molecular feeding analysis are usually fragments of short length with high polymorphism (Shehzad et al. 2012; Thomsen et al. 2012; Bohmann et al. 2014). The sensitivity of these barcodes directly determines the accuracy of species identification in molecular dietary analysis (Sousa et al. 2019). In this paper, we searched key words, such as 'otter' OR '*Lutra lutra*', 'molecular' OR 'barcoding' AND 'diet' OR 'food' OR 'feeding habits' on the ISI Web of Science and Google Scholar online databases and downloaded relevant literature (by July-August 2022). As part of our otter research, we analysed and summarised the efficiency of the barcodes used in the current otter feeding research for species identification and attempted to determine the most suitable molecular method for use in the investigation of otter diet.

DNA amplification and next-generation sequencing for diet analysis

As the primary food source of otters is aquatic vertebrates, we used the vertebrate universal primer pair 12SV5F (5'-ACTGGGATTAGATACCCC-3') and 12SV5R (5'-TAGAACAGGCTCCTCTAG-3'), which are currently the most used in molecular feeding studies of the Eurasian otter (Kumari et al. 2019; Pertoldi et al. 2021), to amplify the mitochondrial 12S rRNA V5 loop fragment of prey DNA from the spraints (Riaz et al. 2011). Although the length of the amplified fragment was only approximately 100 bp, this primer pair has been shown to allow precise discrimination of genus and species across most vertebrate taxa (Sousa et al. 2019; Harper et al. 2020; Zhang et al. 2020; Shao et al. 2021b). Since predator DNA may competitively inhibit the amplification of prey DNA, we added the blocking oligonucleotide OSB1 designed by Kumari et al. (2019) to the PCR to specifically limit the amplification of Eurasian otter mitochondrial 12S rRNA gene. The oligonucleotide was modified to contain a 3-carbon spacer at its 3' end so that DNA replication terminated when the oligonucleotide was bound to the target DNA.

Figure 1. Sampling locations of Eurasian otter spraints in north-eastern China.

All PCR amplifications were conducted in a final volume of 30 µl containing 2 µl DNA extract, 15 µl 2×TransStart FastPfu PCR SuperMix (TransGen Biotech, Inc, Beijing, China), 0.2 µM forward primers, 0.2 µM reverse primers, 2 µM OSB1 and 2 µg BSA (20mg/ml) (TaKaRa, Inc, Dalian, China). The PCR mixture was denatured at 95 °C for 10 min followed by 40 cycles of 30 s at 95 °C for denaturation and 30 s at 50 °C for annealing. Tags, specific for each sample, were added to the 5' ends of the PCR primers (12SV5F/12SV5R). These tags were composed of nine nucleotides with an initial CC followed by seven variable nucleotides. Each tag was designed to differ from the other tags in at least three nucleotide positions; this provided a unique marker for each PCR and permitted precise assignment of sequence reads for relevant samples following NGS. Common carp (*Cyprinus carpio*) DNA (0.08 ng/µl) and Otter tissue DNA (0.08 ng/µl) were the PCR positive controls and the extraction products of the extraction blanks and sterile ionic water were the PCR negative control. All PCRs were performed in triplicate. The products of the replicate PCRs for each sample were mixed and then electrophoresed and visualised on a 2.0% agarose gel. The PCR fragments were extracted from the gel and purified using the AxyPrep DNA gel extraction kit (AxyGen, Inc, CA, USA).

Purified amplicons were quantified using QuantiFluor-ST (Promega, Inc, WI, USA), diluted to 4 nM and then mixed in equimolar concentrations. Each library (containing 109 faecal samples and 4 PCR controls) was paired-end sequenced at 12 pM with one 10% PhiX Control v.3 on the Illumina MiSeq platform (Illumina, Inc, CA, USA) at Shanghai Majorbio Biopharm Biotechnology Co., Ltd. (Shanghai, China) using the TruSeqTM DNA high throughput (HT) library prep kit and the MiSeq Reagent Nano Kit V.2 (300 cycles) (Illumina, Inc, CA, USA) according to the manufacturer's instructions. A total of 150 nucleotides on each of the ends of the DNA fragments were sequenced.

Sequence analysis and taxon assignment

The raw sequences were adapter-trimmed and quality-filtered using the Trimmomatic v.0.39 programme (Bolger et al. 2014). Chimeras were removed using UCHIME (Edgar et al. 2011). Subsequently, we used the OBITools programme to analyse the remaining sequence reads (Boyer et al. 2016). The 'Illuminapairedend' command was used to align and assemble the direct and reverse sequences and unaligned sequences were removed with the 'obigrep' command. Primers and tags were identified in the 'ngsfilter' programme using the criterion of sequences with exact barcode matching and a maximum two-nucleotide mismatch in primers. Identical sequences were combined into a single sequence using the 'obiuniq' programme. Sequence reads with less than 10 occurrences or shorter than 80 bp in length were excluded using the 'obigrep' programme. The 'obiclean' programme was used to detect

and remove erroneous sequences. The remaining sequences were taxonomically assigned by alignment with the available sequences in NCBI using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The bioinformatic processing is fully described in Suppl. material 1: appendix S1.

Due to the existence of a large number of closely-related taxa, species identification, based on sequence similarity, was sometimes ambiguous and duplicates were sometimes observed. To improve the accuracy of taxonomic assignment, we set a threshold that only adopted the query sequences that had 100% coverage in the public database and analysed them using the following criteria: (1) when the percent identity between a query sequence and the reference sequence (refseq) in the database was ≥ 98% and the matched refseq originated only from a single locally occurring species, the query was assigned to that species; (2) when the matched refseq originated from more than one species with ≥ 98% percent identity, the species that were not distributed locally were excluded first; if more than one species then remained, the query was assigned to the lowest taxonomic level that included these species; (3) when the maximum percent identity was < 98% but ≥ 95%, the species identification results were recorded as the lowest taxonomic level that included all of the locally occurring species with the highest identity scores; (4) when the maximum percent identity was < 95%, the taxon could not be classified and was recorded as unknown. If a single non-native species showed the highest identity, the query was assigned to the taxon level of the genus that included the most closely-related native species. The locally occurring vertebrate species were identified by referring to the Illustrated Handbook of Aquatic Animals found at Changbai Mountain (Zheng et al. 1980; Xie 2007). In order to eliminate potential errors caused by tag jumps or cross-contamination, we first merged the duplicate sequences and then removed low frequency sequences including < 0.5% of reads in the PCR or less than the average count of the sequence in the negative control PCRs from the same sequencing library.

Data analysis

We used three formulae to quantify the Eurasian otter diet. Based on the relative frequency of prey in faecal samples, the percent of occurrence (*%POO*) (Xiong et al. 2017) was calculated. The weighted percent of occurrence (*wPOO*) (Tollit et al. 2017) is similar to *POO*, but this metric does not give equal weight to all occurrence events; it weights each occurrence event according to the number of prey species in the sample (e.g. if faecal sample contains three prey items, each item is given a weight of 1/3). The specific formulae used are as follows:

$$\%POO_i = N_i / \sum N_i \times 100\% \quad (1)$$

$$wPOO_i = \frac{1}{N} \sum_{k=1}^{N} \frac{I_{i,k}}{\sum_{i=1}^{T} I_{i,k}} \quad (2)$$

where N is the total number of faecal samples, N_i is the number of faecal samples containing the prey of species i and I is an indicator function such that $I_{i,k} = 1$ if prey Item i occurs in faeces k; otherwise, $I_{i,k} = 0$.

A third formula was used to calculate the relative read abundance (*RRA*) (Deagle et al. 2019) for food items using the sequence counts. This formula is:

$$RRA_i = \frac{1}{N} \sum_{k=1}^{N} \frac{n_{i,k}}{\sum_{i=1}^{T} n_{i,k}} \quad (3)$$

where $n_{i,k}$ is the number of sequences of prey species i in sample k and N is the total number of faecal samples.

Based on the *%POO* data, we used the R package spaa (Zhang 2016) to calculate the dietary parameters related to diversity and niche occupation for the species in the otters' diets. These parameters included Shannon's Diversity Index H, Peilou's J, Levin's niche breadth B and standardised niche breadth Bs. We also used this package to calculate Pianka's Index O to estimate the overlap in the animals' diet between the snow-free season and the snow-cover season and obtained confidence intervals from 1,000 bootstrap samples. To assess whether there were seasonal differences in otter feeding habits, we ran a permutational multivariate analysis of variance (PERMANOVA) using the adonis2 function and checked PERMANOVA assumptions using the betadisper function in the R package vegan (Simpson et al. 2010). Subsequently, 999 permutations were performed to test the similarity percentage (*SIMPER*) of prey composition and to, thereby, determine which prey reflected feeding differences of otters in different seasons. Based on the SPECNUMBER function, we estimated the taxon richness (alpha diversity) of otter diets in different seasons using the R package vegan. Finally, we used species rarefaction and extrapolation curves to estimate the total number of prey species likely to be eaten by the otters in each of the two seasons. These curves for each season were calculated and drawn using the R package *iNEXT* (Hsieh et al. 2016); the number of extrapolated samples used in the calculation was set to 196, the node was set to 20 and the 95% confidence intervals were obtained by 1,000 bootstraps.

Traditional survey of fish biodiversity

In addition, to test the efficiency of otter predation for surveying the environmental fish communities, we set 60 sampling sites in the rivers of HNR (Fig. 1) and investigated fish composition from June to November 2020. Each sampling site was approximately 1000 m in length. For the wadeable streams, electrofishing was applied to collect the aquatic animals. In the unwadeable streams, we used boats to hang seines (30 × 40 mm) for sample collection. Each sample was identified to species by referring to the relevant reference books (Zheng et al. 1980; Xie 2007) and Fish Base Search (https://www.fishbase.se/home.htm). In addition, we also recorded the weight and quantity of fish.

Relative biomass contribution of prey

Due to the difference in prey weight, small prey accounted for more prey biomass (Klare et al. 2011; Wachter et al. 2012). When the frequency method is used to survey the feeding habits of carnivores, the relative importance of large prey in food will be underestimated and that of small prey will be overestimated (Deagle et al. 2019). Therefore, the frequency method alone could not well reflect the relative contribution of each type of prey to the feeding habits of carnivores. To evaluate the relative importance of prey, the relative biomass contribution (*RM*) is commonly used (Klare et al. 2011). To calculate *RM*, we used a formula that is suitable for use in almost all types of carnivores feeding studies (Chakrabarti et al. 2016). The formula is $Y_i = 0.033 - 0.025exp^{-4.284Xi}$, where Y_i is biomass consumed per collected scat/predator weight and *Xi* is prey weight/predator weight. The weight of the predator Eurasian otter is approximately 7.1 kg (Koelewijn et al. 2010). The body weights of some fish were obtained through the fish diversity survey in this study (Suppl. material 2: appendices S2-1, S2-3). The remaining body weights were obtained by consulting the relevant literature. It should be noted that the weights of prey taxa identified to genus or family were obtained by averaging the weights of the most similar species.

Data availability

Raw sequence reads have been archived on the NCBI Sequence Read Archive BioProject: PRJNA908638; BioSamples: SAMN32041161–SAMN32041258; SRA accessions: SRX18492240–SRX18492337.

Results

Of 124 putative Eurasian otter faecal samples collected in the field in the study area (Fig. 1), 115 (92.7%) spraint samples were confirmed by DNA (60 of 60 samples obtained during the snow-cover season and 55 of 64 samples obtained during the snow-free season). Contamination was not observed in the PCR controls. Seventeen samples were excluded due to failure of PCR amplification. The remaining 98 samples (50 from the snow-cover season and 48 from the snow-free season) were subjected to dietary analyses. The sequencing run generated a total of 2,155,491 raw sequence reads and 887,490 sequences were obtained after trimming, merging and length filter application. After removal of chimeras and redundancy via clustering, 884,340 reads remained (average read count of 8,670 per sample including controls), of which 878,486 (99.34%) were assigned a taxonomic rank (Table 1). Each negative control PCR generally exhibited a read length of less than 100 bp, suggesting that contamination was insignificant. Positive control PCRs using otter tissue DNA as the template also exhibited 232 bp reads, indicating that the blocking primer OSB1 was effective. Before threshold application, we detected 36 and 48 discrete species in these two seasons, respectively. After threshold application and elimination of non-native species, we refined the taxonomic assignments; the sequences represented 20 vertebrate taxa, including 13 species, five genera and two families (Table 1). The number of different taxa per faecal sample varied from one to seven (2.4 ± 1.4, $\bar{X} \pm SD$) and 28 of the scats (28.6%) contained only one taxon.

The vertebrate diet of the Eurasian otter

The composition in vertebrates of the diets of Eurasian otters in the HNR was very diverse; it included three mammalian taxa, two amphibian taxa and 15 fish taxa (Table 1). Amongst them, the fish group was captured most frequently by the otters (59.4%). The most frequently occurring taxon was the Northeast forest frog (*Rana dybowskii*; *%POO* = 34.0%), followed by two fishes, the minnow (*Phoxinus*; 15.4%) and the sculpin (*Cottus*; 14.6%) (Fig. 2). At the family level, Cyprinidae (Taxon No. 11-20) was the most frequently occurring prey (36.50%). The most frequent mammalian taxon, the species *Mustela sibirica*, was present in only 2.0% (2/98) of the scats. Only one domestic species was recorded: the domestic pig *Sus scrofa* was recorded in one faecal sample. In addition, three taxa (one mammal and two fishes) appeared only once in otter faeces (Table 1).

Seasonal variations in the otter diet

The Eurasian otters in our study consumed 16 and 14 prey taxa in the snow-cover season and the snow-free season, respectively and there were 10 shared prey taxa (one family, three genera and six species) in the two seasons (Table 1). Prey species unique to the snow-cover season were pigs, bighead Far East goby (*Gymnogobius urotaenia*), Amur goby (*Rhinogobius brunneus*), Amur bitterling (*Rhodeus amarus*), the genus sculpins (*Cottus*), spiny loach (*Cobitis*) and weatherfish (*Misgurnus*). The unique food types in the snow-free season were Siberian weasel (*Mustela sibirica*), Korean field mouse (*Apodemus peninsulae*), eight-barbel loach (*Lefua costata*) and several fishes of the genus *Gobio* (Cyprinidae) (Table 1). The results obtained using *wPOO* and *RRA* to calculate the proportions of various species consumed by otters in the snow-free season, showed that the snow-cover season and the whole year were similar (Fig. 3). The species with the highest *wPOO* and *RRA* values in each season was the Northeast forest frog, which accounted for approximately 50%–60% of the prey consumed. The other two species of prey with high occurrence frequencies in spraints, the sculpin and the minnow, had higher *wPOO* and *RRA* values in the snow-free season than in the snow-covered season. The *wPOO* and *RRA* values of the other species differed little (*F*-test; $p = 0.868$).

Table 1. Vertebrate prey taxa identified in scats of the Eurasian otter (*Lutra lutra*) collected in the in HNR, China.

Prey taxa			Number of occurrences			Number of sequence reads			Most similar sequence(s) and corresponding species in public databases		
Taxon number	Taxon	Common name	Total (n = 98)	Snow-free (n = 48)	Snow-cover (n = 50)	Total (n = 98)	Snow-free (n = 48)	Snow-cover (n = 50)	Scientific name	Identity (%)	Accession number
	Mammals										
	(1) Artiodactyla										
1	*Sus scrofa*	Pig	1	0	1	171	0	171	*Sus scrofa*	100	MH603005
	(2) Carnivora										
2	*Mustela sibirica*	Siberian weasel	2	2	0	38195	38195	0	*Mustela sibirica*	100	MN206976
	(3) Rodentia										
3	*Apodemus peninsulae*	Korean field mouse	1	1	0	2567	2567	0	*Apodemus peninsulae*	100	AJ311142
	Amphibian										
	(1) Anura										
4	*Rana dybowskii*	Northeast forest frog	82	41	41	486829	350150	136679	*Rana dybowskii*	98.88	KF898355
5	*Glandirana emeljanovi*	Northeast China rough-skinned frog	12	2	10	11811	364	11447	*Glandirana emeljanovi*	100	KF771343
	Osteichthyes										
	(1) Perciformes										
6	*Perccottus glenii*	Chinese sleeper	4	2	2	10422	8695	1727	*Perccottus glenii*	100	KC292213
7	*Gymnogobius urotaenia*	Big head Far East goby	3	0	3	489	0	489	*Gymnogobius urotaenia*	100	KT601093
8	*Rhinogobius brunneus*	Amur goby	2	0	2	240	0	240	*Rhinogobius brunneus*	100	KT601096
	(2) Scorpaeniformes										
9	*Cottus*	Sculpin	35	21	14	213750	200620	13130	*Cottus szanaga/ Cottus czerskii/ Cottus poecilopus*	100	KX762050/ KJ956027/ AB188185
	(3) Gasterosteiformes										
10	*Pungitius sinensis*	Nine-spine stickleback	11	4	7	10395	1011	9384	*Pungitius sinensis*	98.99	MF990245
	(4) Cypriniformes										
11	*Cobitis granoei*	Siberian spiny loach	16	2	14	15218	538	14680	*Cobitis granoei*	100	MN153552
12	*Lefua costata*	Eight-barbel loach	1	1	0	293	293	0	*Lefua costata*	97.98	KT943751
13	*Barbatula*	Tone loach	10	6	4	4060	3399	661	*Barbatula nuda/ Barbatula toni*	100	KF574248/ MK900633
14	*Cobitis*	Spiny loach	4	0	4	2581	0	2581	*Cobitis granoei / Cobitis lutheri*	97.98	KF908768/ AB860297
15	*Misgurnus*	Weatherfish	2	0	2	13596	0	13596	*Misgurnus bipartitus/ Misgurnus anguillicaudatus/ Misgurnus mohoity*	100	KF562047/ EU670804/ KF386025
16	*Phoxinus phoxinus*	Tumen hill-brook minnows	6	2	4	2669	1736	933	*Phoxinus phoxinus*	100	KC992395
17	*Rhodeus amarus*	Amur bitterling	4	0	4	839	0	839	*Rhodeus amarus*	100	AP011209
18	*Phoxinus*	Minnow	37	24	13	54542	37405	17137	*Phoxinus lagowskii / Phoxinus percnurus*	100	KR091310/ AP009061
19	Cyprinidae 1	Gudgeon 1	7	1	6	9122	491	8631	*Gobio cynocephalus/ Saurogobio dabryi*	100	KU314700/ KF534790
20	Cyprinidae 2	Gudgeon 2	1	1	0	697	697	0	*Hypophthalmichthys molitrix/Elopichthys bambusa/Squalidus chankaensis*	99	MF180232/ KM196112/ MK840863

The dietary overlap between seasons was very high (Pinka'O = 0.91, 95% CI 0.62–0.98) and the prey taxa were significantly positively correlated (Spearman rho = 0.59, $p < 0.01$). The Shannon Diversity Index values, Peilou's Evenness, dietary niche width and standardised dietary niche width in the snow-cover season were higher than those in the snow-free season (Table 2). The PERMANOVA analysis, based on the adonis2 function, showed that there were significant differences in the food items consumed by otters in the two seasons (marginal $R^2 = 0.032$, $p = 0.009$). The results of the Betadisper function analysis confirmed that PERMANOVA did not show homogeneity of variance ($p = 0.039$). The results of *SIMPER* analysis showed that the average contributions of the five prey groups sculpin, minnow, Northeast forest frog, Siberian spiny loach (*Cobitis granoei*) and Northeast China rough-skinned frog (*Glandirana emeljanovi*) to the seasonal feeding differences of otters were highest, but only the contributions of minnow ($p = 0.021$) and Siberian spiny loach ($p = 0.013$) were significant (Table 3).

The rarefaction/extrapolation (R/E) curves showed that, under the condition of the existing sample size, the prey item richness curve in the two seasons had reached a stable level. The sample coverage was 96.50% and 99.03% in the snow-cover season and the snow-free season, respectively (Fig. 4).

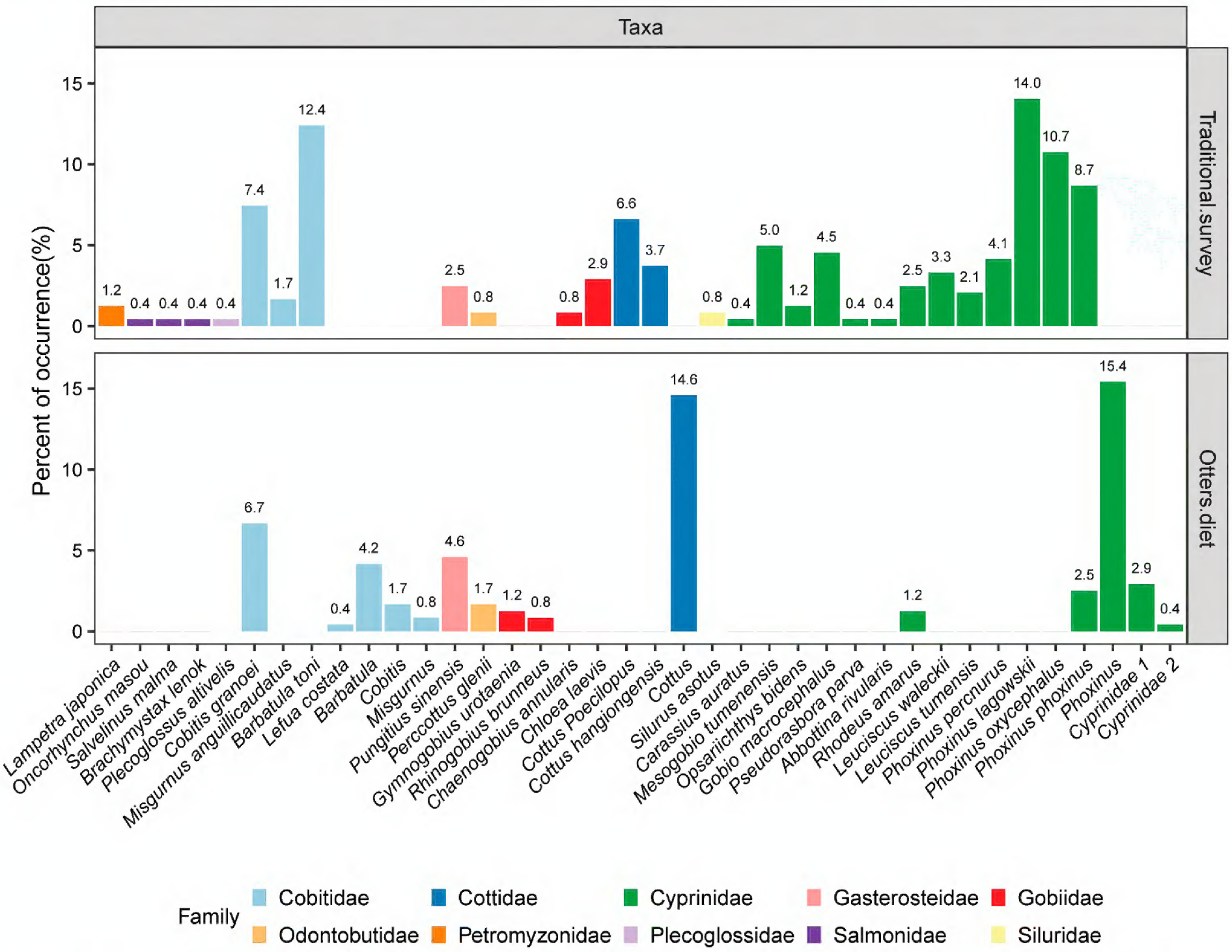

Figure 2. Bar plot showing the percentage occurrence (%*POO*) of fish taxa captured using traditional methods and molecular otter feeding analysis, respectively. The numbers on the bars represent the %*POO* values for each taxon. Bars are coloured according to fish family.

Comparison of the fish composition of otter diets with the results of traditional fish surveys

According to historical survey data and records (Zheng et al. 1980), there may be 68 species of fish (belonging to 16 families and 45 genera) distributed in local rivers in the study area (Suppl. material 2: appendix S1). Through the traditional survey method, we caught 4,021 fish with a total weight of 31.17 kg at 60 sampling points in the study area (Suppl. material 2: appendix S2-1). Based on morphological analyses, 28 species of fish accounting for 41.2% of the recorded fish were confirmed (Fig. 2). The number of fish species identified at each sampling point ranged from one to 11 (5.6 ± 2.5, $\bar{X}$± *SD*) (Suppl. material 2: appendix S2-2) and the average weight of the individuals of each fish species ranged from 0.9 to 100.4 g (19.4 ± 24.0, $\bar{X}$± *SD*) (Suppl. material 2: appendix S2-3). Amongst the detected fishes, the family Cyprinidae represented the largest number (73.7%) and the most species (46.4%).

The diet composition analysis showed that the collected otter faeces contained fish belonging to 15 taxa represented by two families, five genera and eight species (Table 1). At the species level, the traditional fish survey detected five species with average weights ranging from 0.9 g to 48.3 g (15.1 ± 18.1, $\bar{X}$± *SD*); these five species accounted for 17.86% of the fish detected in the traditional survey (Fig. 2, Suppl. material 2: appendix S2-1). The other three fish species, which included the eight-barbel loach, the bighead Far East goby and the Amur goby, were not captured in the traditional survey (Fig. 2).

Table 2. Statistics of prey taxa diversity and niche width of Eurasian Otter in different seasons.

Metrics	Total	Snow-cover	Snow-free
H	2.19	2.30	1.83
J	0.73	0.83	0.70
B	5.71	6.74	4.36
B_S	0.25	0.38	0.26

Table 3. The SIMPER analysis results of prey composition in otter diet. The Table shows the five prey groups with the highest average contributions to the seasonal feeding differences of otters.

	Average contribution (%)	*sd*	Cumulative contribution (%)	*p*
Cottus	0.106535	0.12552	0.1957	0.234
Phoxinus	0.103967	0.11307	0.3866	0.021*
Rana dybowskii	0.059669	0.10615	0.4962	0.604
Cobitis granoei	0.046745	0.07463	0.582	0.013*
Glandirana emeljanovi	0.040878	0.07987	0.6571	0.057

Note: Snow-cover vs. snow-free Average Dissimilarity = 0.54.

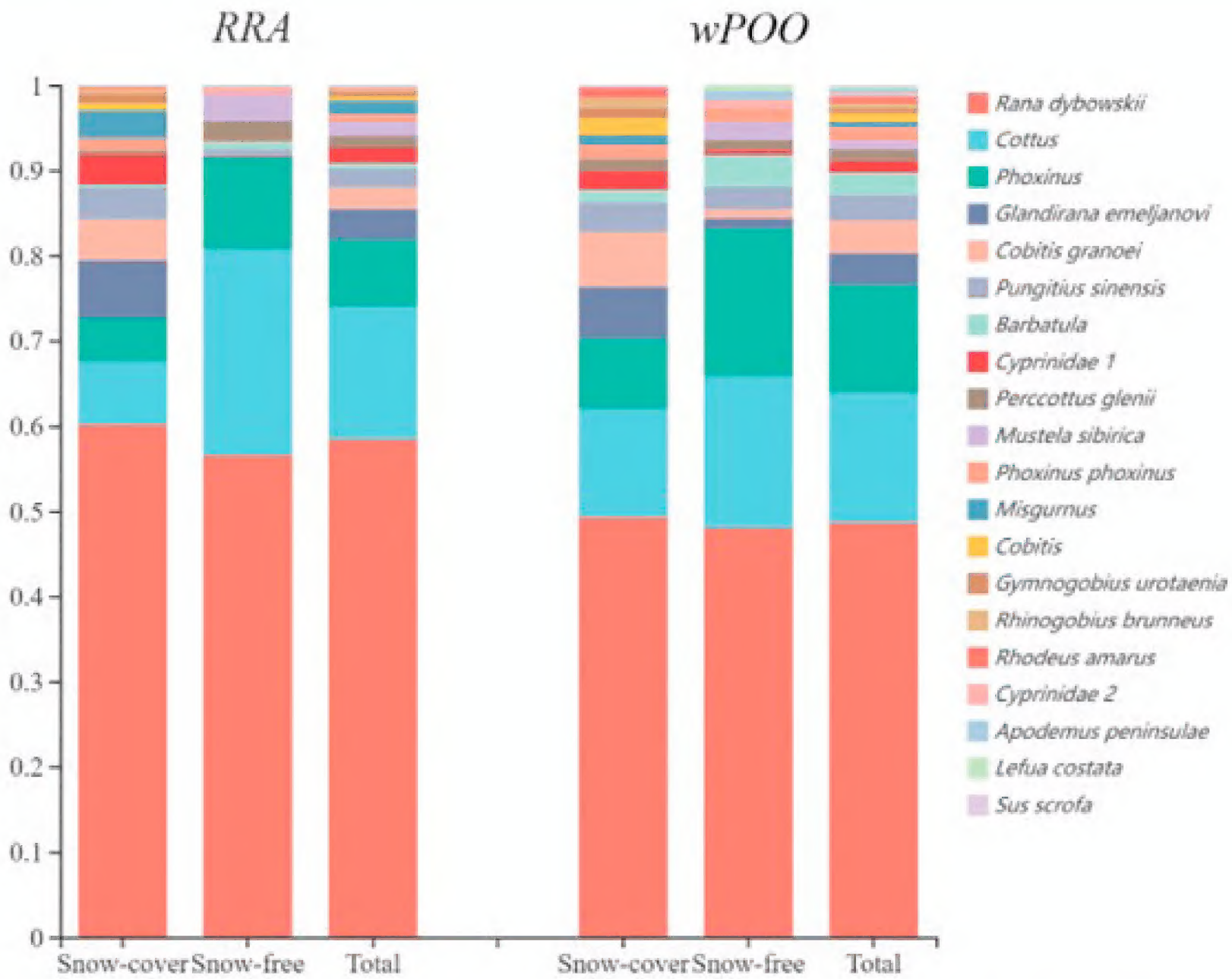

Figure 3. Comparison of the relative read abundance (*RRA*) and weighted percent of occurrence (*wPOO*) in the diet of the Eurasian otter in one year, snow-cover and snow-free season. The three columns on the left represent the values of *RRA* and the right represent the values of *wPOO*.

At the genus and family levels, the seven prey taxa that were found to be present in otter diet may include some of the 16 local fish species that were obtained by sequence alignment (Table 1, Suppl. material 2: appendix S3). Five of these 16 fish species were also captured in the traditional survey, whereas the remaining 11 species were not (Suppl. material 2: appendix S3).

Relative biomass contribution of prey

The relative biomass contribution results showed that fish is the primary vertebrate food source for otters (57.4%), followed by amphibians (38.9%) and mammals (3.7%) (Table 4). Amongst all prey taxa, the Northeast forest frog had the highest *RM* value for otters (34.1%), followed by sculpin (14.5%) and minnow (14.3%). At the family level, Cyprinidae (Taxon No. 11-20) had the highest *RM* value (35.1%).

Efficiency of species resolution in the evaluation of otter feeding habits

Eight studies of the diet of Eurasian otters that used molecular dietary analysis were found by searching online databases through late December 2021. These studies were conducted in five countries and published between 2019 and 2021 (Table 5). The barcodes used for prey species identification in these studies were fragments of animal DNA with lengths of 36 ~ 650 bp. Six of the studies used only one barcode for vertebrate identification in otter faeces and the other two studies used three and nine barcodes for the identification of vertebrates and other prey groups, such as insecta, crustacea and malacostraca (Fig. 5).

The number of taxa identified in these studies ranged from 4 to 76 (27.9 ± 21.3, $\bar{X} \pm SD$) (Fig. 5) and the proportions of taxa which were identified at the species level were 25% ~ 100% (67.6% ± 23.7%, $\bar{X} \pm SD$) (Table 5). Compared with these studies, based on the number of taxa identified (20) and the species identification rate (65.0%), the efficiency of our identification of species consumed by otters was at the average level (Fig. 5).

Discussion

Diet of the Eurasian otter

The results of our study on the diet of otters in the HNR of northeast China show that fishes are the main vertebrate prey category and amphibians are the secondary prey category of Eurasian otters in this region; this is consistent with previous findings for temperate Europe and neighbouring South Korea (Clavero et al. 2003; Krawczyk et al. 2016; Hong et al. 2019; Kumari et al. 2019; Buglione et al. 2020). Studies on otter feeding habits

Table 4. Prey species composition of otter diet and their percent of occurrence (%*POO*), estimated average weight and relative biomass contribution (%*RM*) in HNR, northeast China.

Taxon number	Taxon	Common name	*POO* (%)	Average weight (kg)	RM (%)
1	*Sus scrofa*	Pig	0.41	81.540	1.56
2	*Mustela sibirica*	Siberian weasel	0.83	0.850	1.73
3	*Apodemus peninsulae*	Korean field mouse	0.41	0.033	0.40
4	*Rana dybowskii*	Northeast forest frog	34.02	0.045	34.10
5	*Glandirana emeljanovi*	Northeast China rough-skinned frog	4.98	0.025	4.82
6	*Perccottus glenii*	Chinese sleeper	1.66	0.021	1.60
7	*Gymnogobius urotaenia*	Big head Far East goby	1.24	0.024	1.20
8	*Rhinogobius brunneus*	Amur goby	0.83	0.001	0.77
9	*Cottus*	Sculpin	14.52	0.041	14.45
10	*Pungitius sinensis*	Nine-spine stickleback	4.56	0.001	4.23
11	*Cobitis granoei*	Siberian spiny loach	6.64	0.003	6.18
12	*Lefua costata*	Eight-barbel loach	0.41	0.002	0.38
13	*Barbatula*	Tone loach	4.15	0.005	3.87
14	*Cobitis*	Spiny loach	1.66	0.004	1.55
15	*Misgurnus*	Weatherfish	0.83	0.011	0.78
16	*Phoxinus phoxinus*	Tumen hill-brook minnows	2.49	0.003	2.32
17	*Rhodeus amarus*	Amur bitterling	1.66	0.048	1.67
18	*Phoxinus*	Minnow	15.35	0.005	14.33
19	Cyprinidae 1	Gudgeon 1	2.90	0.018	2.77
20	Cyprinidae 2	Gudgeon 2	0.41	2.420	1.29

suggest that geographical climate is an important reason for plasticity in otter feeding behaviour. For example, the diet of Eurasian otters in Mediterranean Europe is more diverse than that of the same species in northern temperate Europe (Clavero et al. 2003; Remonti et al. 2008). In Mediterranean localities, the resources for selectable prey in the environment are extremely broad (e.g. crabs, crayfish, amphibians and insects) and the relative proportion of fish consumed decreases, especially during dry periods; at such times, otters turn to other prey items to compensate for the scarcity of fish (Román 2011). In temperate Europe, the primary and favourite vertebrate prey of Eurasian otters is fish, followed by amphibians (Clavero et al. 2003; Krawczyk et al. 2016; Harper et al. 2020); this is consistent with our observation of otters' diet in the HNR. The HNR has a temperate Asian climate and the similarity of situation and species composition is the main reason for the similar prey selection behaviour of otters living at both ends of Eurasia.

Habitat characteristics can also affect the prey composition of otters. For example, in Poland, otters caught more fish in standing water than in flowing water (Krawczyk et al. 2016). In central Finland, amphibians were found to be an important prey of otters that inhabit woodland streams (Sulkava 1996). In our study, the Northeast forest frog was the prey species with the highest frequency of occurrence (*%POO* = 34.0%) and highest relative biomass contribution (%*RM* = 34.1%), indicating that the Northeast forest frog is the key prey species for otters in the HNR, which is a typical mountain forest. Traditional Chinese medicine holds that the gonads of Northeast forest frogs help fight off illness and nourish the body; therefore, there is a huge consumer market in China for this product (Huang and Bai 2003). The HNR is an important area of production of Northeast forest frogs and a large

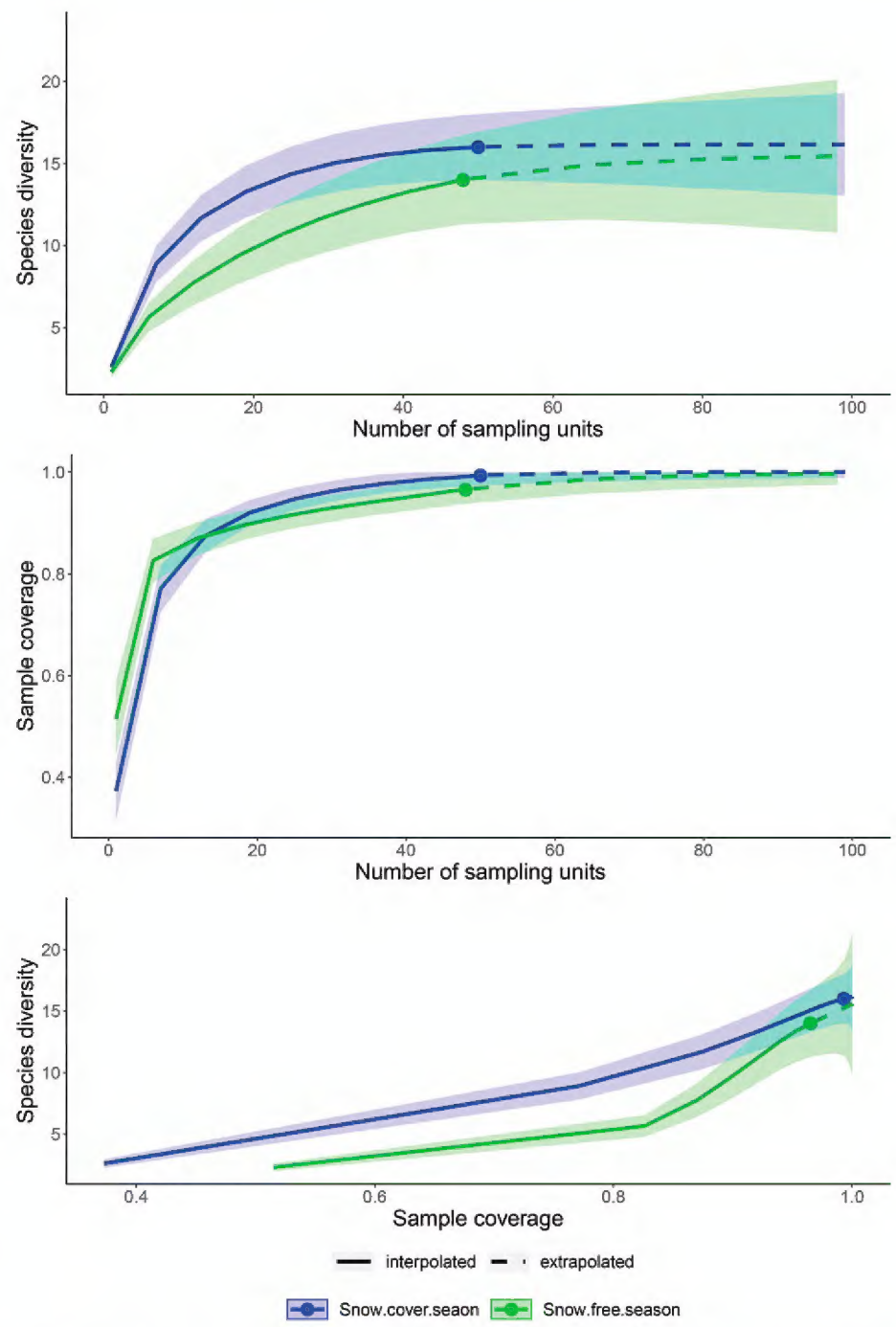

Figure 4. Rarefaction and extrapolation curves produced for Eurasian otter scats from HNR in snow-cover and snow-free season using iNEXT. Figure on the top represents sample size-based R/E curve, Figure in the middle represents sample completeness curve and Figure at the bottom represents coverage-based R/E curve.

Table 5. Comparison of DNA barcodes and species resolution in molecular dietary investigation of Eurasian otter around the world.

Location	Sample No.	Primer name	Target gene	Target animals	Sequence length (bp)	Prey items (taxon No. were identified to species level)	References
China	98	12SV5F/12SV5R	12S rRNA	Vertebrate	~ 100	20 (13)	Present study
Taiwan, China	64	BirdF1/ BirdRM	CO I	Birds	650	16 (14)	Jang-Liaw et al. (2021)
		VF1/VRM	CO I	Mammals, reptiles, fish, amphibians, and some insects	650		
		chmf4/chmr4	CO I	Amphibians	650		
		FF2d/ FR1d	CO I	Fishes	650		
		FishF1/ FishR1	CO I	Fishes	650		
		FishF2/ FishR2	CO I	Fishes	650		
		LCO1490/ HCO2198	CO I	Various phyla from the animal kingdom	650		
		LepF1/ LepR1	CO I	Lepidoptera	650		
		Til9020F/ TilMR	COIII	Tilapia	254–548		
Denmark	212	12SV5F/12SV5R	12S rRNA	Vertebrate	~ 100	35 (27)	Pertoldi et al. (2021)
Italy	49	16Smam_1/16Smam_2	16S rRNA	Vertebrate	140	21 (12)	Buglione et al. (2020)
England	171	12SV5F/12SV5R	12S rRNA	Vertebrate	~ 100	37 (32)	Harper et al. (2020)
Italy	50	1391F/1795R	18S rRNA	Vertebrates and Decapoda	160–170	4 (1)	Marcolin et al. (2020)
Spain	50	Teleo-12SF/Teleo-12SR	12S rRNA	Fish	418–636	7 (7)	Martínez-Abraín et al. (2020)
South Korea	7	12SV5F/12SV5R	12S rRNA	Vertebrate	~ 100	28 (17)	Kumari et al. (2019)
		VF2/FishF2/ FishR2/FR1d	CO I	Fish	~ 631		
		16SMAVF/16SMAVR	16S rRNA	Invertebrate	~36		
South Korea	24	MT0698L/MT1076H	12S rRNA	Vertebrate	400	76 (34)	Hong et al. (2019)

number of open-air frog breeding ponds without fences have been built near the streams in the HNR; this has undoubtedly greatly improved the availability of Northeast forest frogs and made it easier for otters to prey on them.

Previous studies have confirmed that Eurasian otters consume fewer prey items in stable habitats on the regional scale (Lanszki and Molnar 2003; Baltrunaite 2009). Our research supports this assertion. We discovered that the prey composition of otters included fewer taxa in the snow-free season than in the snow-cover season. Apparently, because of the lower prey availability, otters expanded the spectrum of prey utilised to maintain normal biomass intake in the snow-cover season. Moreover, when in suboptimal circumstances, otters usually shift their prey preference from fish to other prey. For example, Brzeziński et al. (1993) and Sulkava (1996) found that the percentage of amphibians in the diet increased in winter, mainly because hibernating or spawning frogs are easily caught by otters. However, our study found no obvious increase in foraging on amphibians in the snow-cover period. This may be because many of the amphibians in the environment, mainly Northeast forest frogs, were harvested by farmers before the frogs' hibernation period, resulting in a decline in amphibian availability.

Mammals and birds are a tertiary significant prey of otters and the percentage of these animals in otter diets usually presents seasonal variation (Lanszki and Sallai 2006; Krawczyk et al. 2016). In our study, domestic pigs were only detected in the diet of otters during the snow-cover season. On further investigation, we found that farmers often place dead pigs in frog breeding ponds to help the frogs and their tadpoles survive the cold winter. This might cause the otters' faecal DNA to contain the DNA of domestic pigs. Diet analysis also confirmed

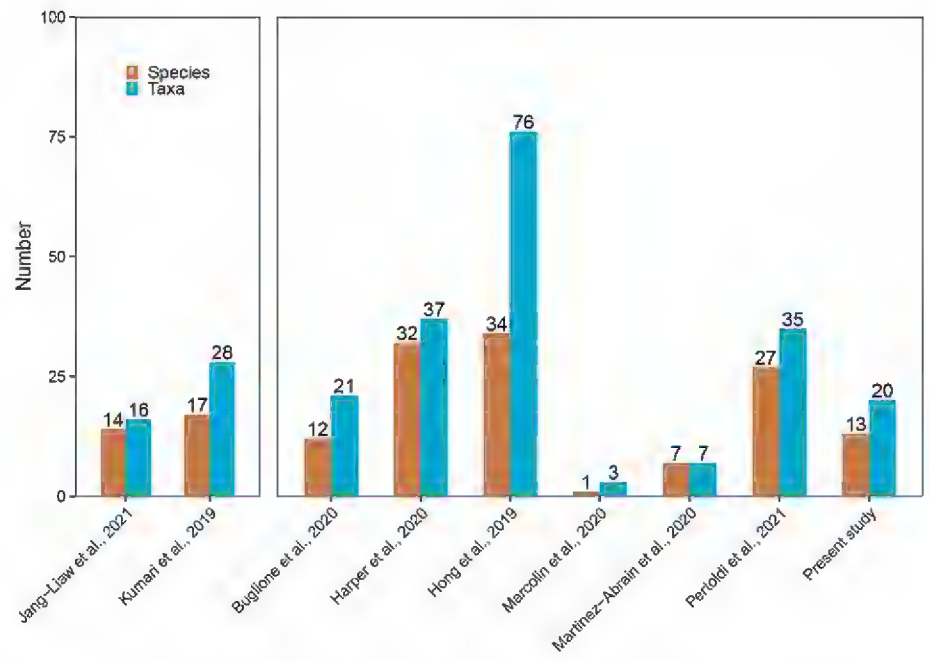

Figure 5. Number of prey taxa and identification to species level taxonomic units detected by molecular dietary investigation of Eurasian otter around the world. The left and right bar charts showing the results of multi-barcodes and single-barcode survey, respectively.

that DNA from the Siberian weasel and the Korean field mouse, two typical forest animals, appeared in otter faeces during the snow-free season. Using an infrared camera trap network set in the HNR, we obtained a large number of videos of otters moving in relatively high-altitude areas, such as hillsides and ridges, during snow-free season. To some extent, the feeding habits of carnivores reflect the spatiotemporal overlap between predators and prey (Kronfeld-Schor and Dayan 2003). Based on this, we hypothesise that the spatial activity of otters is driven by prey and that their key prey may be the Northeast forest frog. The seasonal activities of the Northeast forest frog are quite regular and can be divided into four periods: uphill into the forest, life in the forest, downhill

out of the forest and hibernation (Huang 2007). From the beginning of May, the Northeast forest frog migrates to the mountains and then enters its forest life period. By the end of September or early October, the frogs come out of the forest and gradually migrate along relatively fixed routes (such as gullies and streams) to the lowland near the river, where they enter their hibernation period when the temperature falls below 10 °C. This frog migration may lead to an increase in the frequency of otter activity in the forest and mountains during the snow-free season. The emergence of these two wild mammals in spraints might be the result of opportunistic predation by Eurasian otters or simply be these mammals interacting with otter faeces in some way, for example, sniffing, urinating or defecating on top of them.

Efficiency of the molecular analysis of otter feeding habits in surveying environmental fish communities

In terms of the high amphibian composition of the diet of otters, our study area appears to host an abundant population of amphibians, especially species of Northeast forest frogs. It has been demonstrated that amphibians are less preferred than fish as prey due to their lower energy value (Krawczyk et al. 2016). Eurasian otters usually feed on amphibians when fish are scarce (Lanszki and Sallai 2006; Sittenthaler et al. 2019; Martínez-Abraín et al. 2020); thus, we speculate that the availability of fish might be relatively lower in the HNR than in other areas. We believe that, in addition to the naturally lower abundance and richness of fish populations in woodland streams, another reason for the scarcity of fish may be the large-scale cultivation of Northeast forest frogs; such cultivation seriously depletes the habitats and resources available to fish, eventually resulting in the reduction of local fish populations.

Compared with the results of investigations conducted using traditional fishing methods, few fish (eight species) were identified at the species level through DNA analysis of the otter diet (Fig. 2). Three of these species were not detected using traditional fishing methods. The remaining seven fish taxa in the otter diet were not successfully identified at the species level. If DNA barcodes with higher discriminatory power can be developed for these seven taxa in the future and all species of these taxa can be identified, the number of identified species may increase by 7 ~ 16. In addition to the eight species previously identified, there are at least 15 ~ 24 species of fish in the otter diet; this is close to the number of species identified in traditional fish surveys and roughly reflects the species composition of local aquatic fish. However, with the current barcode recognition rate, investigation of fish diversity using molecular analysis of the otter diet alone is obviously not an ideal approach. Nevertheless, considering the natural advantage this approach offers for the identification of secretive and scarce species, we suggest that molecular diet surveys can be used as an auxiliary means of investigating fish diversity.

Species resolution of otter molecular diet analysis

Sequence divergence of the taxa that contribute to food composition restricts the species resolution and accuracy of DNA-based diet surveys. Due to the high plasticity of Eurasian otter predatory behaviour, the dietary components of otters living in disparate habitats vary considerably (Remonti et al. 2008; Krawczyk et al. 2016). In this case, even if the same barcode markers are used in different studies of otters' diets, the species resolution achieved usually differs. For example, Harper et al. (2020) used the same DNA barcode that we used in this study to survey the food composition of otters in England and 32 of the 37 prey taxa were successfully identified at the species level. However, our study identified 13 of 20 species, a lower level of species resolution. The probable cause for this is that many closely-related species, many of which are members of the Cobitidae and the Cyprinidae, appeared in the otter diet in our study and this made accurate classification difficult.

In addition to the complexity of prey composition, the sensitivity of detection of prey DNA using DNA barcodes is another factor that affects the species resolution of food taxa. In reviewing previous studies in which molecular analysis of the otter diet was conducted, we found that 14 of 16 primers were used in the species identification of vertebrates (Table 5). Of these primers, universal primers for all types of vertebrates (e.g. 12SV5) were the most commonly used, while only five fish-specific primers were applied in three studies, which may affect the species resolution of fish that have been proved to be an important prey of Eurasian otters (Lanszki and Sallai 2006; Buglione et al. 2020; Harper et al. 2020). This suggests that the primers currently used in molecular diet surveys of Eurasian otters are not necessarily the most suitable. Quéméré et al. (2021) used the "12S-Teleo" primers specifically designed for fish DNA metabarcoding to investigate the diet of Giant otters (*Pteronura brasiliensis*) and achieved > 90% fish species resolution rate. Miya et al. (2015) provided a set of universal PCR primers (MiFish) for metabarcoding eDNA from fish. Due to its high efficiency in fish species resolution, it has become the most used method/primers for fish metabarcoding worldwide (Weigand et al. 2019; Miya et al. 2020). These primers could be considered for future studies on feeding habits of Eurasian otters. Besides, appropriate barcodes not only improve the species resolution, but also avoid species identification mistakes. Havmøller et al. (2020) used both COI and 16S rRNA as target regions to survey the African leopard diet. Whereas the result showed that five samples yielded 16S OTUs with 96% similarity to the published DNA reference sequence of yellow baboon (*Papio cynocephalus*), three of these samples were found to have COI sequences with 100% similarity to published sequences of kipunji monkey (*Rungwecebus kipunji*). Currently, many studies have used generic primers directly to monitor environmental biodiversity with little knowledge of local species composition, which is not rigorous. Even if some primers showed a high species

identification rates, there is a risk of misidentifying some species as the same species due to the absence of differences in the target regions of the barcodes, leading to an underestimation of species richness. To solve this problem, selecting the right primers and using simultaneously several primer pairs may be a relatively effective approach (Elbrecht et al. 2016; Tournayre et al. 2020).

The sensitivity of species identification is also dependent on the integrity and quality of the reference database used in molecular dietary analysis and extensive coverage of the potential prey can greatly facilitate species-level identification (Xiong et al. 2017). For example, Hong et al. (2019) successfully identified 76 taxa in the diet of otters in South Korea; this is much higher than the number of taxa identified in other molecular dietary studies of otters. In their study, Hong et al. (2019) first investigated the presence of specific fish species in the environment using scoop nets and casting nets and, thereby, established a reference database of potential otter prey, based on survey data. A combination of morphological examination and molecular identification of faecal content was then used to increase the number of taxa identified and the accuracy of the identification. Simultaneous integration of analyses of the feeding habits of otters with prey investigation can also provide additional information, such as estimates of fish size, prey selection, spatio-temporal changes in feeding habits and competitor interactions, that can enhance the comprehensive understanding of otter foraging strategies. In this study, we searched the 12S rRNA gene sequences from NCBI for historically-distributed fish (Zheng et al. 1980; Xie 2007) and fish captured by our traditional survey and the results showed that most fish sequences (92.6%) could be retrieved, which could exclude the theory that the low species resolution was due to the lack of reference sequences.

Implications for conservation

Our study reveals the highly diverse feeding habits and versatile foraging skills of Eurasian otters. A high percentage of the Eurasian otter's diet was found to consist of fish, suggesting that Eurasian otters may play a significant role in controlling fish populations in the freshwater ecosystem of northeast China. In addition, some land mammals appear in the otter diet and infrared camera data also indicate that otter activities extend to mountain forests. The spatial distribution and functions of this semi-aquatic animal in terrestrial ecosystems require further investigation. Generalist carnivores can be effective samplers of the biodiversity of regional vertebrates, based on the premise of highly efficient species resolution and wide coverage through diet metabarcoding analysis (Shao et al. 2021a). Although the molecular approach offers remarkable taxonomic discriminatory power, it should be applied with caution and sophisticated and specialised surveys are needed as a basis for the use of the method, especially when it is used to explore in detail the food network structure in highly complex ecosystems, such as our study area, in which closely-related prey species co-exist. In this case, our suggestion is that the procedure followed in molecular dietary analysis should consist of two steps. The first step involves the use of appropriate DNA barcodes to roughly identify large prey classes (e.g. the COI, 12S or 16S rRNA gene barcode for vertebrates and the 16S gene barcode for invertebrates, Table 5); the second step consists of the development and utilisation of specific primers for fine identification of taxa that were not identified at the species level in the first step.

Variations in prey communities can be triggered by anthropogenic drivers (Zhang et al. 2020; Consuegra et al. 2021). Poaching, intensive hunting and habitat manipulation can decrease prey availability, posing significant threats to carnivore populations and affecting their feeding habits (Shao et al. 2021b). The high proportion of Northeast forest frogs in the food habits of otters in the HNR suggests that human interference has had a great impact on otters. The free-ranging frogs accounted for a significant proportion of the environmental resources and reduced the abundance and diversity of other prey (Huang 2007). Otters prey heavily on forest frogs due to a lack of other food and this results in financial damage to farmers. To prevent the otters from catching cultured frogs, farmers set traps around the frog ponds, further aggravating the conflict between farmers and otters. In October 2021, the Northeast Tiger and Leopard National Park, covering an area of 14,600 km^2 in northeast China, was officially established and the HNR was incorporated and managed. In the future, policies designed to strictly control human activities will be established in the area and animal husbandry and agriculture, which have been conducted there for decades, will be entirely banned. This means that the Northeast forest frog, an important source of food for otters in this area, may decrease in number and this will almost certainly result in fluctuations in the otter population. Therefore, the management department should conduct a long-term investigation of the population dynamics of otters and their prey to ensure that the otters can maintain stability and health.

Conclusion

Environmental DNA metabarcoding technology has become the most popular technology in biodiversity surveys with high efficiency, accurate results and simple operation. Amongsr them, the high species-level resolution of barcodes is an important reason for the success of this technology. However, our research found that barcodes, which are commonly used at present, are not suitable for all environmental species diversity surveys, especially when there are many related species in the environment, the taxonomic resolution of barcodes will be greatly reduced. Based on this, we suggest that, when using eDNA combined with metabarcoding to investigate species diversity in the future, in addition to using barcodes with high universality, it is also necessary to use specific molecular sites to identify those closely-related species, so as to improve the efficiency of species identification.

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Supplementary material 1

Bioinformatic processing

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Data type: docx. file

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Supplementary material 2

appendices S1–S4.2

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Data type: tables (excel file)

Explanation note: appendix S1. List of recorded fish species in the HNR, China. appendix S2-1. Species, number, total weight, and average weight of fish determined from traditional capture approaches in HNR, China. appendix S2-2. Species and number of fish determined from traditional capture approaches in HNR, China. H* represents the sampling site. appendix S2-3. Species and total weight of fish determined from traditional capture approaches in HNR, China. H* represents the sampling. appendix S3. Fish species determined from traditional capture approaches in HNR, China (black circle), fish taxa determined from molecular dietary. appendix S4-1. Coordinates of aquatic resources investigation site in HNR, China. appendix S4-2. Sampling locations of Eurasian otter spraints in snow-free season. appendix S4-3. Sampling locations of Eurasian otter spraints in snow-cover season.

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